PATENT

Attorney Docket No. 16243-1-5

#30 MSW 9/21/94

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of:

Richard H. Tullis

Serial No.: 08/078,767

Filed: June 16, 1993

For: OLIGONUCLEOTIDE THERAPEUTIC AGENT AND METHODS OF MAKING SAME

Examiner: J. Martinell

Art Unit: 1805

DECLARATION PURSUANT TO 37 C.F.R. § 1.132

Hon. Commissioner of Patents
and Trademarks
Washington, D.C. 20231

Sir:

I, Dr. Jerry L. Ruth, being duly warned that willful false statements and the like are punishable by fine or imprisonment or both, under 18 U.S.C. § 1001, and may jeopardize the validity of the patent application or any patent issuing thereon, state and declare as follows:

Considered 11/2/94

1. All statements herein made of my own knowledge are true and statements made on information or belief are believed to be true. The Exhibits (1-8) attached hereto are incorporated herein by reference.

2. I received a Ph.D. in synthetic organic chemistry from the University of California at Davis in 1978.

A copy of my curriculum vitae is attached as Exhibit 1.

3. I am presently employed at the U.S. Fish and Wildlife Forensics Laboratory where I am primarily responsible for conducting DNA analysis to assist in the identification of wildlife for threatened and endangered species of animals. Prior to my obtaining employment at the Forensics Laboratory, I was a vice president of research at Molecular Biosystems where my responsibilities involved the development of nucleic acid based diagnostic kits.

4. I have read and am familiar with the contents of the application and related papers. I understand that the Examiner has made two rejections. The first rejection is for failure to fully teach how to make and use the invention as claimed, and the second is that the invention is obvious over a combination of four references. This declaration will address both rejections. I will provide objective evidence of the state of the art of arresting specific protein expression by oligonucleotide hybridization in 1981. The evidence will be provided by interpretation of references relating to this art and by my personal perspective from having been involved in the field of oligonucleotide chemistry and biology in 1981.

5. **ENABLEMENT**

A. **Analogs of Nucleic Acid**

It is my understanding that the Examiner has concerns that the invention should be limited to phosphotriester-modified nucleic acid and that claims reading on natural nucleic acid and other analogs are not enabled. It is my opinion that once Dr. Tullis identified functional sizes and the mRNA coding region as a target, the invention was fully disclosed to one of skill. The utility of natural nucleic acid and various analogs to be internalized by cells and inhibit cell function was known. Evidence of these facts can be found in the prior art. For example, Miller (1977) describes neutral, nonionic nucleic acids for nonspecific inhibition of protein synthesis. Befort (1974) used methylated ribonucleic acid to inhibit viral replication. Zamecnik and Stephenson (1978) used a natural phosphodiester DNA tridecamer to inhibit viral replication *in vivo*. Finally, Summerton (1979) described a number of early reports using various nucleic acid analogs to inhibit viral infections (see page 89).

B. **Making Ribonucleic Acid**

The Examiner further argues that there is no teaching of how to make or use ribonucleotides. The methodology for making synthetic RNA and its analogs was available in 1981. Evidence of this fact can be found in the Miller reference (1977). Therein the authors used an analog of ribonucleic acid to non-specifically inhibit protein synthesis.

Moreover, methods for chemical synthesis of oligoribonucleotides were well established by 1981 as is illustrated by reference to the work of Ohtsuka

and his colleagues. In the mid and late 1970s, Ohtsuka's group reported synthesis of numerous oligoribonucleotides corresponding to the sequence of an *E. coli* tRNA. Some of this work is described in *Nuc. Acids. Res. Symp. Series (NARS)* No. 7, pp. 335-343 (1980), which is attached as Exhibit 3, and the cites therein. In 1980 Ohtsuka reported synthesis of oligonucleotides corresponding to the total sequence of *Escherichia coli* $tRNA_f^{met}$; these oligos were joined using RNA ligase to create an entirely synthetic tRNA (*Id.*). The synthesis of *E. coli* tRNA is also discussed in a subsequent paper that appeared prior to the filing date of the application [Proc. Nat. Acad. Sci. 78(9) 5493 (1981); attached as Exhibit 4]. Clearly, by 1981 methods for chemical synthesis had been available for several years.

Enzymatic methods for synthesis of oligoribonucleotides complement chemical methods and include use of polynucleotide phosphorylase and T4 RNA ligase. RNA ligase in particular has been useful in synthesis of oligoribonucleotides and in 1980 Gumport *et al.*, in a paper on T4 RNA ligase, observed that "...the enzyme is now widely used to synthesize defined sequences of RNA." [NARS No. 7 (1980) pp. 167-171 at 167; attached as Exhibit 5]. In a 1981 review attached as Exhibit 6, ("T4 RNA Ligase as a Nucleic Acid Synthesis and Modification Reagent" in *Gene Amplification and Analysis*, Vol. 2, Chirikjian and Papas, eds. Elsevier (New York) 1981, pp. 314-345 at pages 335-339), Gumport and Uhlenbeck describe work by several groups engaged in oligoribonucleotide synthesis, including Ohtsuka [described above], Neilson and colleagues [using a combination of organic and enzymatic methods to prepare several decanucleotides], Krug and colleagues [preparation of a 21-nucleotide RNA], and others [see citations at 337, first full paragraph]. In my opinion, the attached exhibits clearly demonstrate that methods for synthesis of oligoribonucleotides were well known in 1981.

C. <u>Cell Uptake of Nucleic Acid</u>

Finally the Examiner raised the issue of cell uptake of nucleic acid. He comments that there are no data and methods for actually "getting short DNAs or RNAs into cells." Living cells have a natural capacity for internalizing short nucleic acids. There is nothing to teach. The cells internalize these compounds without

special culture conditions. There is no need to render the cells porous. The literature relied upon by the Examiner to support his obviousness rejection teaches this fundamental fact. For example, the Miller reference involves the effect of a trinucleotide analog on mammalian cells and the Summerton reference discloses at pages 93-94 the routine uptake by animal cells of both RNA and DNA. Finally there is the paper by Zamecnik and Stephenson (1978) which describes the internalization of viral infected cells by a DNA of 13 nucleotides.

6. **OBVIOUSNESS**

It is my understanding that the Examiner believes that in 1981 a person of skill reading Itakura *et al.*, Paterson *et al.* or Hastie *et al.* and Summerton or Miller *et al.* would have had a motivation and a reasonable expectation that targeting the coding region of a specific mRNA with a oligonucleotide complementary to the coding region would have arrested protein translation of that mRNA. There are a number of objective reasons why this is not an accurate statement of the state of the art in 1981.

A. <u>The secondary structure of the mRNA made it an unlikely target for control of expression by complementary oligonucleotides.</u>

The claimed invention went against the conventional wisdom of the time. The conventional wisdom in 1981 was that the secondary structure of mRNA was extensive that there was no reasonable likelihood that a oligonucleotide complementary to a coding sequence would have sufficient access to arrest translation inside a living cell. In addition, those of skill understood that the natural mechanism of peptide elongation by ribosomes involved the destabilization of the extensive secondary structure of the mRNA. For these reasons, the idea of hybridizing a complementary oligonucleotide to a coding region of mRNA to arrest translation was contrary to conventional wisdom. The oligonucleotide would have to overcome two significant hurdles. First it had to bind to the coding region of the mRNA, which was viewed as a Gordian knot of secondary structure. And even if the complementary oligonucleotide could find and anneal to its complementary subsequence, the ribosomes were viewed as able to read mRNA coding regions constrained by extensive secondary structure. Thus it was not likely that the

hybridization of a complementary oligonucleotide would arrest translation. The ribosome would simply "toss" the duplexing structure aside.

Although there is little express language in the prior art articulating the above concerns, the literature indirectly compels one to conclude that secondary structure and ribosomal helix destabilization were concepts teaching away from the invention. References published after the priority date of the subject application do expressly identify the state of the art at the time of the invention.

The extensive secondary structure of mRNA was well understood in 1981. In the book, The Ribonucleic Acids, 1977, Eds. Stewart and Latham, Springer Verlag, Chpt. 4, "Messenger RNA" by J.M. Adams, the extensive secondary structure in phage mRNA and eukaryotic mRNA is described. Those of skill recognized the importance of mRNA secondary structure and particularly were aware of the need of low mRNA secondary structure in the regions where ribosomes initially bind to mRNA. W. Salser, in his chapter Globin mRNA Sequences: Analysis of Base Pairing and Evolutionary Implications, in *Chromatin* Vol XLII (1978) pages 985-1001 at page 992, provided a graphic illustration of the extensive amount of secondary structure in the typical mRNA. In Dr. Salser's illustration, the sequences involved in ribosome initiation and interaction are dramatically free of secondary structure. Dr. Adams, on page 103 in his section (e) entitled "Influence of secondary and tertiary structure on initiation," also discusses the importance of secondary structure on initiation of translation. In chapter 7, by Wettenhall and Clarke-Walker of *The Ribonucleic Acids*, the authors on page 258 in a section entitled "Messenger RNA structure" expressly state, "[t]he role of mRNA structure in initiation reactions is receiving considerable attention." These authors also discuss the role of secondary structure of mRNA on translation efficiency.

The concern over availability of the coding region to bind oligonucleotides is made more apparent when one looks at the secondary references relied upon by the Examiner. These references are Paterson *et al.* and Hastie *et al.* describing cell-free, *in vitro* experiments in which denaturing conditions to relax the secondary structure of their mRNA are applied prior to hybridizing nucleic acids. For example, Patterson used 100°C for 30 seconds and

Hastie used temperatures between 45°C and 65°C. For the Examiner to believe that one of skill would have understood that complementary oligonucleotides were able to bind to the coding regions of mRNA under *in vivo* conditions when both Hastie and Paterson used denaturing conditions is logically inconsistent and scientifically incorrect.

A number of other references taught that the targeting of a coding regions would not be a preferred target for a oligonucleotide agent expected to control expression. Pluskal *et al. Biochem. Soc. Trans. 7*:1091-1093 (1979), wrote that their work with a heterogenous mixture of low molecular weight oligonucleotides non-specifically inhibited translation, but that the mode of action was not affected by preincubation with the mRNA. One of skill is left to conclude that the translation inhibition observed by Pluskal did not occur via complementary binding interactions between the oligonucleotides and the mRNA.

Continuing in temporal order, the Ts'o patent further suggests away from the Applicant's invention. In the Ts'o patent, the claimed oligonucleotide analogues are described as inhibiting expression of a preselected sequence, either a cellular nucleic acid or a viral nucleic acid. Ts'o uses trimer analogues and at column 25, lines 5-10, they state that their analogues inhibited poly(U) messages but not globin messages. In column 26, lines 5-34, Ts'o explains that they saw no inhibition *in vivo* of either bacteria or hamster cells.

Ts'o's results are consistent with later published reports. These later reports actually explain what was intuitively apparent to those of ordinary skill at the time Dr. Tullis filed his application in 1981. There was no objective reason or basis to conclude that the coding region of mRNA was an effective target for *in vivo* control of expression by trinucleotides because there was no evidence that the trimers used by Ts'o inhibited elongation when bound to mRNA either *in vitro* or *in vivo*. Either the coding regions of mRNA were physically unavailable to complementary oligonucleotides or even if binding to the coding regions, the oligonucleotides were unable to arrest the elongation process of the ribosomes. As explained above, the latter concept was particularly compelling because to elongate, ribosomes have to untwist (denature) the secondary structure of mRNA.

Perhaps the most express and compelling articulation of the true state of the art from 1977 until after the effective filing date of the subject application is found in Dr. Miller's own work published in two parallel reports in 1985. In Blake, Murakami and Miller, *Biochemistry 24*, 6132-6138 and *24*, 6139-6145, the authors discuss at length their concerns over secondary structure on the availability of subsequences of mRNA to complementary oligonucleotides. In the first of the two companion articles (A22), Dr. Miller looked at 8-12 mer oligonucleotides and concluded, on page 6135, that in the cell free rabbit reticulocyte protein expression system, 8-mer oligonucleotides do not arrest protein elongation, but that if bound to the initiation codon region the oligonucleotides will arrest translation. On the second column of page 6136, Dr. Miller in summarizing the state of the art expressly states:

> The above results are in agreement with the recent findings of Liebhaber *et al.* (1984). They found that cDNAs to human globin mRNA which cover the initiation codon or extend into the 5'-noncoding region are able to completely inhibit translation in a rabbit reticulocyte lysate. In contrast cDNAs which cover only the coding region exclusive of the initiation site are not effective at blocking translation. These authors postulate that a helix-destabilizing activity associated with the reticulocyte ribosomes is able to disrupt secondary structure during the elongation step but not the initiation step of translation. **Thus, cDNAs or oligonucleotides bound to the coding region of mRNA would be expected to be unable to prevent translation in the reticulocyte system.** [Emphasis added]

Dr. Miller is summarizing two points to explain why oligonucleotides binding to the initiation codons will inhibit translation but those binding to coding regions will not inhibit translation. He first explains that the ribosomes will simply remove the oligonucleotides bound to the translated region (page 6136, column 2), and second, he explains that the secondary structure of mRNA precludes the binding of the oligonucleotides to the translated region (page 6137). He implies this is true even for cDNAs which may be hundreds of bases long.

In the second article, Dr. Miller is again reporting on the ability of oligonucleotides to arrest *in vitro* translation. In this report the oligonucleotides are between 6 and 11 bases long. The authors are reporting some degree of success with the binding of oligonucleotides to mRNA coding regions. Although the

success of the method is being reported, the authors are clearly articulating the concerns over secondary structure which was the conventional wisdom in 1981. For example at page 6144, the authors state:

> In addition to the effect of mRNA secondary structure, the region of the mRNA to which the oligonucleotide methylphosphonates binds influences its effectiveness as an inhibitor of mRNA translation. The results in Table II suggest that oligomers complementary to the 5' end and initiation codon regions are somewhat better inhibitors than the oligomers which bind to the coding regions. **Thus it appears that the initiation step of translation is more sensitive to oligomer mRNA binding than the elongation step of translation.** [Emphasis added]

Finally, as late as 1986, the literature was still suggesting that short oligonucleotides will only arrest translation if bound to the initiation sites. In particular, Haeuptle *et al.,* using a cell free translation system, clearly states that size of the oligonucleotides bound to coding regions is a critical key to ensuring arrest of translation. Just as is taught by the subject application with its effective filing date of 1981, Haeuptle reports in 1986 that the oligonucleotides must be 10 bases or greater in length before substantial inhibition of translation is demonstrated. The Examiner should also note that the authors, in deference to secondary structure, are using 55°C for 5 minutes to relax the mRNA sufficiently to allow the oligonucleotides to bind to their target regions.

In conclusion, I state that the conventional wisdom in the art, at the time of the invention, taught away from Dr. Tullis' invention. The literature cited above is offered as evidence of this wisdom. This literature clearly teaches that the secondary structure of mRNA, especially in the coding region, was thought by those of skill in 1981 to be extensive and that this secondary structure would preclude binding of a complementary oligonucleotide to the coding region mRNA for the purpose of arresting translation. Also, there was reason to believe that the helix destabilization of ribosomes during elongation would have easily displaced any oligonucleotide duplexed on the coding region. Therefore, one of skill would not have considered the claimed invention to have a reasonable expectation of success in 1981 when Dr. Tullis originally filed the subject patent application.

B. <u>The Examiner's interpretation of the text in Miller (1977) goes well beyond the understanding one of skill would have reading the same text in 1981.</u>

It is my further understanding that the Examiner has stated that Miller (1977) expressly states that oligonucleotides complementary to the coding region of mRNA might inhibit cellular protein synthesis *in vivo*. The two statements have been cited by the Examiner. The abstract states:

> Our biochemical studies suggest that inhibition of cellular protein synthesis might be expected if $G^mp(Et)G^mp(Et)U$, $G^mp(Et)G^mpU$, and G^mpG^mpU, which have been taken up by or formed within the cell, physically bind to tRNA and mRNA and inhibit the function of these nucleic acids.

The last paragraph states:

> The triester $G^mp(Et)G^mp(Et)U$ is complementary to target nucleic acids involved in cellular protein synthesis and has a transitory effect on the cells which is then relieved by its degradation. Such a transitory effect of inhibition may provide a convenient and useful way of imposing a temporary interruption of cellular functions. On the other hand, neutral oligonucleotide analogues with longer chain lengths and a greater resistance to hydrolysis would be expected to achieve a greater specificity and effectiveness in regulating the function of target nucleic acids inside the cell over a long period of time. The observations of the present study lay the groundwork for continued investigation into the use of neutral oligonucleotide analogues as proves and regulators of nucleic acid function within living cells.

There are multiple reasons why one of skill in 1981 would not have interpreted the cited text in the way the Examiner suggests. The text clearly does not state that oligonucleotide analogs could bind to the coding regions of mRNA. At best it is ambiguous when taken out of context both literally and historically. It is literally ambiguous because the authors' work is directed to general inhibition of all protein expression by using a trimer to bind to the amino acid accepting codon of a tRNA. There is no evidence of trimer binding to target mRNA or of any inhibition of specific protein expression due to complementary binding of Miller's trimer to mRNA.

The phrase "greater specificity" is patently ambiguous. Statistically, a trimer sequence is represented every 64 nucleotides and therefore a timer binds

non-specifically. Miller's use of "greater specificity" could refer to oligonucleotides that are specific only for the amino acid accepting codon of tRNA or that will bind specifically (only) to the initiation region of a mRNA, or thirdly, as the Examiner would read the passage, using longer oligonucleotides that would bind with greater specificity to the coding region of an mRNA.

In historical context, the meaning of the above quoted text is clearly directed to either the tRNA or mRNA binding sites. These were only regions perceived by scientists in 1981 as being sufficiently accessible to complementary oligonucleotide binding under *in vivo* conditions. As explained above, the extensive secondary structure of mRNA was a primary reason why those of skill would have avoided the coding region of mRNA to arrest translation. For example, in Dr. Salser's review article of 1978, he includes a figure (Figure 3) depicting the proposed secondary structure of a mRNA. It is an extraordinary complex and twisted structure. Only a few regions are accessible to a complementary oligonucleotide and these are designated as points of initiation and tRNA interactions. These open regions which are generally found as single-strand hairpin loops.

Furthermore, the historical record indicates that by April of 1981, Dr. Mill understood the limitations of his 1977 report. In *Biochemistry*, **20**:1874-1880, Exhibit 8, Dr. Miller *et al.* reported on the arrest of globin expression via blockage of tRNA aminoacylation by trimers and tetramers. On page 1879, and at Table VI, they report no effect on globin synthesis despite the fact that the oligonucleotides used by Miller (polyA) could bind to at least three sites in the coding region of mRNA encoding globin (codons 45, 85 and 118). See Exhibit 9.

Finally, in 1985, the last two Miller references summarize the historical evidence that taught away from targeting the coding regions of mRNA with oligonucleotide to arrest translation. First, in the introductions to both 1985 papers, there is no reference to the 1977 paper when discussing mRNA as a target for control of protein expression by complementary oligonucleotides. Secondly, as stated above on pages 7-8, the 1985 references specifically suggest; (1) that secondary structure will prevent binding of oligonucleotides to mRNA; and, (2) that helix destabilizing properties of ribosomes will remove oligonucleotide even it they

were to have access to the coding region of an mRNA. These are two conclusive reasons why one of skill would not expect the arrest of protein translation by oligonucleotide binding to the two conclusive coding regions of mRNA.

In summary, by 1981, Miller's reference to "greater specificity" would not have been interpreted as a suggestion for controlling the expression of particular "target" proteins by binding to specific coding regions of mRNA, but as a suggestion to use longer oligonucleotides to bind **specifically** to the open regions of the tRNA or mRNA that bind to rRNA during the initiation step of synthesis. The open binding sites are longer than three bases and thus one would expect greater specificity for binding by using oligonucleotides greater than three bases. For these reasons, I state definitively that one of skill reading Miller in 1981 would have recognized that the proposed targets for binding oligonucleotides are these binding sites.

To argue that in 1981, one of ordinary skill would have predicted *in vivo* utility for an oligonucleotide binding to the coding portions of mRNA is to read Miller in a vacuum, and ignores the authors' data and the historical understanding of the accessibility of the coding region of mRNA due to secondary structure.

C. Although the Examiner reads the Miller reference as suggesting the use of an oligonucleotide binding to the coding region of an mRNA, Miller does not suggest this aspect of Dr. Tullis' invention.

The Miller reference is silent as to the target sequences on a mRNA to which its oligonucleotides might bind. Messenger RNAs have several functional domains. The coding region is only one domain. These various domains are described as "signals" by W. Salser in his chapter Globin mRNA Sequences: Analysis of Base Pairing and Evolutionary Implications, in *Chromatin* Vol XLII (1978) pages 985-1001. The signal sequences are described on page 988 as including "ribosome binding, mRNA processing, transport from the nucleus to the cytoplasm and so on".

Dr. Tullis' claims involve only the coding region and one reading Miller would not have been directed to this region. One of skill with knowledge of the secondary constraints of the coding region of mRNA and the mechanism by which

ribosomes read mRNA would not have read Miller as suggesting the targeting of the mRNA coding region.

D. **Even if the prior art oligonucleotides of Miller were binding to the coding region of an mRNA, they could not have arrested translation.**

As further evidence that the Examiner's interpretation of Miller goes beyond any reasonable interpretation of the reference by one of skill in 1981, I would like to point out that the inhibition detected by Miller was in fact solely due to a non-specific interference of tRNA. We know this because Miller uses only trimer oligonucleotides and trimers were later shown to be useless as inhibitors of translation even if bound to a coding region of an mRNA.

The Examiner is directed to the 1986 reference of Haeuptle *et al.* Haeuptle describes the *in vitro* arrest of translation by oligodeoxyribonucleotides. The authors relaxed the secondary structure of mRNA encoding lysozyme using 55°C and hybridized oligonucleotides of varying lengths to a portion of the coding region. On page 1435 and in Figure 4 on page 1439, they present evidence of their inability to arrest translation using short oligonucleotides of 5 bases. The 5-mer species are simply too small to have sufficient binding strength to arrest the progress of a ribosome. The authors reported successful arrest of translation with their 10, 15 and 20 mer species.

E. **The Examiner has misinterpreted the use of "specificity" by Miller in 1977.**

The Examiner relies on the final paragraph of Miller, 1977 stating: "... oligonucleotide analogs with longer chain lengths and a greater resistance to hydrolysis would be expected to achieve a greater *specificity* and effectiveness in regulating the function of target nucleic acids...". The Examiner reads this sentence to include a suggestion to use oligonucleotides for longer binding to the coding regions of mRNA. Above I have explained that this phrase is and would have been understood by one of skill in 1981 to be directed to the non-coding regions of mRNA. I would like to focus this part of my declaration on evidence that the authors' reference to "specificity" was in a general context and did not refer to the coding regions of mRNA encoding specific proteins. More particularly,

the authors were referring to oligonucleotides that bound more specifically to the amino acid accepting end of the tRNA and the initiation sites of mRNA.

Dr. Miller's later publication, Blake *et al.* (1985A) provides evidence of his intended meaning in using "specificity" in 1977. On page 6137, column 2 is the word "specific[1]" used in the same context that the word "specificity[2]" was used in 1977. Miller is again asserting that his work suggests *specific* control of expression by binding oligonucleotides to mRNA. But the context of the 1985 reference clearly implies that the arresting oligonucleotides are not directed to the coding regions of the mRNA. The Blake article states that the only available regions are the initiation points due to secondary structure. Thus the Examiner's interpretation of the text of Miller (1977) in an unlimited and broad context so as to include the coding regions of mRNA is to ignore the scientific realities of the contemporaneous literature.

F. <u>There are objective scientific reasons why one of skill would have not had a reasonable expectation that oligonucleotides complementary to the coding region of an mRNA could arrest translation of a specific protein.</u>

The examiner apparently believes that the cell-free systems of Hastie and Patterson provide one of skill with a reasonable expectation that *in vivo* arrest of protein expression was possible with oligonucleotides. This is not true. Both Hastie and Patterson used cDNA of lengths that exceed 500 bases. These DNAs required harsh denaturing conditions to effect binding to mRNA. The *in vivo* activity of oligonucleotides which are short DNA species, preferably between 13 to 23 bases, cannot be predicted by the *in vitro* behavior of cDNA. Furthermore the cDNA used by both Hastie and Patterson could not be used for *in vivo* arrest of protein expression. They are too long to cross a cell membrane.

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[1] Dr. Miller states in Blake *et al.* at page 6137, column 2, "The results of this study show that sequence-specific oligodeoxynucleotides can be used to arrest translation of specific mRNAs in a selective manner in cell-free systems."

[2] At page 1995, Miller states: "neutral oligonucleotide analogues with longer chain lengths and a greater resistance to hydrolysis would be expected to achieve a greater specificity and effectiveness in regulating the function of target nucleic acids inside the cell over a long time period."

In addition to the impropriety of comparing cDNA to oligonucleotides, there are a variety of additional objective reasons why there was no reasonable expectation in 1981 that oligonucleotides complementary to the coding region of mRNA could arrest translation of specific proteins *in vivo.* The osmotic potentials, the salt and pH conditions, the microenvironments within the various compartments of the cells, the ability of oligonucleotides to bind divalent cations and affect the electropotential and homeostasis of a cell, the secondary structure of the mRNA *in vivo*, the physical pressure and gel-like consistency of the cytosol, are all factors which precluded the ability of one of skill to predict with a reasonable certainty that complementary oligonucleotides could bind with sufficient strength, if at all, to the coding regions of mRNA to selectively arrest translation. As Dr. W. Salser succinctly stated in his 1978 review article, "The problem is then to assess which parts of the [mRNA] structure in Figure 3 may actually exist in the complex milieu of the cell."

Furthermore, even if one were to ignore the conventional wisdom that the secondary structure of mRNA rendered the coding region inaccessible to complementary oligonucleotides, there were a number of unknown parameters which alone or in combination render the question of successful regulation of expression by the claimed methods unpredictable. More specifically, there were a number of valid reasons why the oligonucleotides expected to arrest translation might not physically reach the mRNA inside a living cell. The mRNA of eukaryotes is produced in membrane bound nuclei. The art demonstrated that oligonucleotides would be taken up by cells, but not whether the oligonucleotides would be allowed access to the internal regions of the nuclei. Befort *et al.*, at page 184, states that exogenous oligonucleotides used as antiviral agents enter cells but are primarily bound to the microsomal fractions and do not significantly enter the host cell nuclei. It was known that messenger ribonucleic acid proteins bound to mRNA. According to Pain and Clemens, in *Comprehensive Biochemistry*, Vol 19B, Part 1 at pages 14-15 (1980), the function of these proteins was unknown. These proteins might have played a role in the storage and delivery of the mRNA to the correct ribosomes (membrane bound or not) and might have interfered with the access of the mRNA complementary oligonucleotides. As the authors go on to explain, only

10% of the total mRNA produced by the cells is actually used by the ribosomes to produce protein. That unknown mechanism, perhaps controlled by messenger ribonucleoprotein, as well as other unpredictable aspects of mRNA transport, could very well have rendered the mRNA unaccessible to complementary oligonucleotides.

In addition, there were the unknown effects of spermidine and spermine. These common polyamines tightly bind to the phosphate backbone of mRNA *in vivo* and play a role in the structure and function of mRNA at the ribosomes during protein synthesis. The impact of spermidine on the ability of oligonucleotides to bind *in vivo* to mRNA, either because the polyamines binding to the oligonucleotides might prevent hybridization to mRNA, or because spermidine bound to mRNA might block oligonucleotide binding *in vivo*, was simply unknown. Thus, the effect of spermidine and spermine was yet another unknown factor that would lead one of skill away from a reasonable expectation that one could effect the *in vivo* arrest of translation by oligonucleotides complementary to the coding region of specific mRNA.

In conclusion, it is clear that one of skill would not have expected with any reasonable degree of certainty in 1981 that an oligonucleotide specific for the coding region of an mRNA could arrest translation. In summary, the following seven objective reasons were identified above: (a) that the intact mRNA might not be physically accessible to complementary oligonucleotides; (b) that secondary structure of mRNA might block complementary oligonucleotide binding; (c) that short oligonucleotides might not have sufficient binding strength to block a ribosome designed to untangling internal duplexes in mRNA; (d) that the use of unsuitably long complementary oligonucleotides would have their own secondary structure that would interfere with hybridization of mRNA; (e) that polyamines and transport proteins might have rendered the coding region of mRNA inaccessible; (f) that the ability of oligonucleotides to bind cations might have had a toxic effect on target cells; and (g) that the majority of mRNA are not actually translated by cells but rapidly turned over.

This Declarant has nothing further to say.

Dated: 23 August 1994

Jerry L Ruth

Jerry L. Ruth, Ph.D.

attachments:

Exhibit 1 [Ruth C.V.]
Exhibit 2 [Schwartz C.V.]
Exhibit 3 [Ohtsuka *et al.* 1980]
Exhibit 4 [Ohtsuka *et al.* 1981]
Exhibit 5 [Gumport *et al.* 1980]
Exhibit 6 [Gumport and Uhlenbeck 1981]
Exhibit 7 [Miller *et al.* 1977]
Exhibit 8 [Miller *et al.* 1977]
Exhibit 9 [Efstratiadis *et al.* 1977]

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EXHIBIT I

California Biomedical Research Foundation
DEPARTMENT OF BIOLOGICAL CHEMISTRY
741 Arenas Street
La Jolla, California 92037
Telephone (714) 454-8571

Dear Bill,

It was a pleasure talking to you on the phone about my idea for a new type of antibiotic employing synthetic oligonucleotides specific for particular mRNA's. Especially in finding that you had attended UCSD, knew Paul Price and had taught O-Chem with him. I am looking for to meeting both you and Mr. Kouelman. Since it's getting late now I'll get down to business.

The idea is as follows:

① Synthetic oligodeoxynucleotides can be now manufactured which bind specifically to particular mRNAs, forming an RNA-DNA hybrid.

② mRNA-DNA hybrids cannot be translated into proteins

③ Oligonucleotides are taken up by cells and will form RNA-DNA hybrids under physiological conditions (ie 37 C; ca 0.2 M salt)

Therefore it is possible to make specific oligonucleotides which will selectively block any selected organism from growing. (eg block a virus growing inside a cell)

EXHIBIT O

This system is not relegated to viral infections alone. Any organism (such as an insect, a malarial parasite or a leprosy causing bacterium) which can be made to take up the oligonucleotide can be selectively killed. Thus this system represents an entirely new class of antibiotics which can in theory be directed at any organism.

I have considered several possibilities with regard to this system which might occur as objections against it. I will deal with these in order of importance.

① Is this system sufficiently selective?

The best evidence experimentially is that a 12 base oligodeoxynucleotide designed to specifically bind to gastrin mRNA can select it out of a population of about 30,000 different sequences which are present in stomach A^+ RNA!

Another way to view the same question is to ask how many possible sequences are there in any cell? In general there are about 10,000 different mRNA sequences per cell each about 1000 bases long. Thus the sequence complexity is about 10^7 bases or nucleotides (NT). For a 15 NT oligomer there are 4^{15} different possibilities. Thus the chance of a random matchup between any particular mRNA and a specific 15 NT oligomer is

$$\frac{4^{15}}{10^7} = \frac{1.07 \times 10^9}{10^7} \simeq 100:1$$

As you increase the size of the oligonucleotide, the chance of a random hybridization decreases exponentially

$4^{12} = 17 \times 10^6$ $\quad$ $4^{15} = 1.07 \times 10^9$

$4^{13} = 67 \times 10^6$ $\quad$ $4^{16} = 4.3 \times 10^9$

$4^{14} = 268 \times 10^6$ $\quad$ $4^{17} = 17 \times 10^9$

Thus it is possible to design an oligonucleotide which is extremely specific for a particular mRNA, and therefore for a specific organism.

② Will the system work in a living situation

The growth of RSV (Rous sarcoma virus) can be selectively blocked in cultured cells with no evidence of toxicity to the cells. by a specific oligonucleotide. The treatment also blocks the ability of the virus to transform the cells into cancer cells.

③ What do I propose thats unique?

The design of particular oligodeoxynucleotides (synthesized as phosphotriesters) which inactivate organisms other than RSV. (eg malaria)
Phosphotriester formation has two beneficiall effects

④ it makes the oligonucleotide virtually immune to degradation by the cell

(b) it makes the oligonucleotide soluble in lipid phases (eg membranes) while preserving its ability to bind to RNA and form hybrids. This should allow it easy access to the interior of the cell, as shown in the Too paper.

I hope this short explanation is sufficient. I have done many other calculations, but I see no reason to go into them at this time. Please let me know what else you require.

Best Regards

Richard H. Jullis.

PS.

PPS. This idea first occurred to me during a conversation with Ken Widder (Keudrew Biosystems) in Long Beach in the afternoon of . . .

EXHIBIT II

Dear Bill,

I have been trying to break loose some time to consider further some thoughts about polynucleotides as antibiotic agents. What comes out of that has been two ideas which are really partly questions. (I'm thinking as I go here)

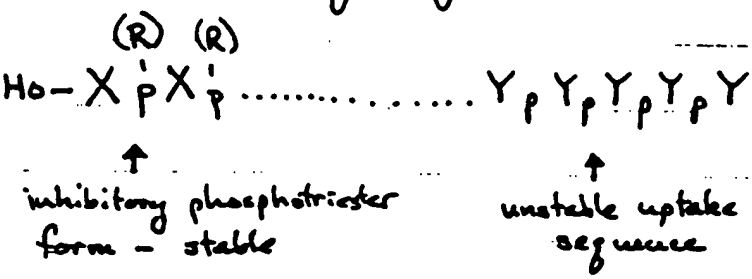
① How well does an oligonucleotide get into a cell if it is synthesized as a phosphotriester or left charged. ?

② How soluble are phosphotriesters and would that be a problem for either injecting them; clearance by white blood cells, kidney, etc; and would they get to the right place

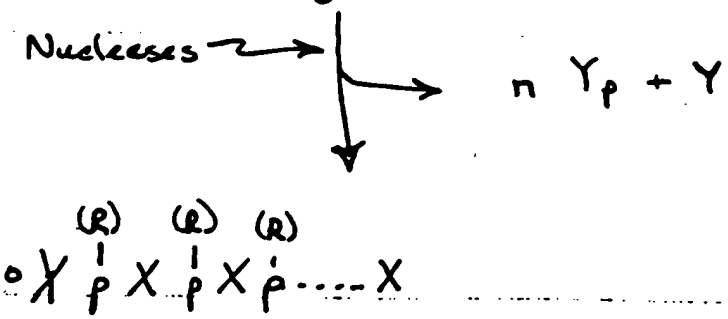
③ It is known that oligonucleotides of 2'→5' phosphodiester linkages are stable to nucleases. Might use that in place of phosphotriester to stabilize the inhibitor. Problem is that 2'→5' oligo's may not form stable duplex structures - that's not known yet.

④ There may be sequence selectivity to cellular uptake of DNA molecules. This is true for Haemophilus influenzae. If so you could add that sequence

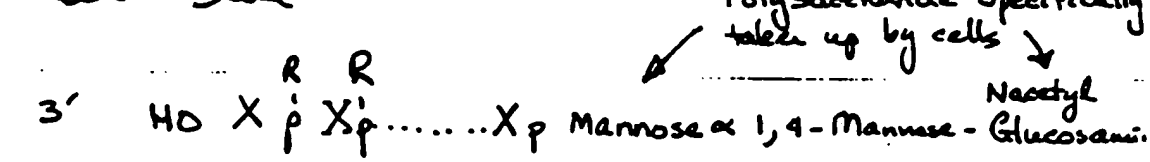
as a relatively unstable oligonucleotide on the 5′ side of the inhibitory oligonucleotide.



When this gets taken up into a cell the uptake signal sequence can be cleaved leaving the more stable inhibitory sequence

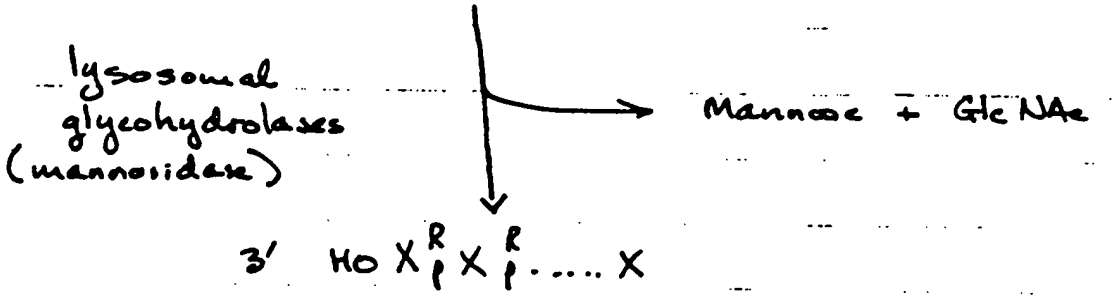


This idea could be extended to other types of labile carriers. I don't at this point know enough about uptake of molecules to know the best ones but one such carrier might look like



Once again cells (mammalian cells this time – fibroblasts, liver cells) have specific uptake systems for non-sialyat polysaccharides with a high mannose content

Therefore this material should be taken up just like asialoglycoproteins by pinocytosis into (ultimately) lysosomes where the sugar is degraded leaving the oligonucleotide which can escape to the rest of the cell and subsequently block translation of its target mRNA.



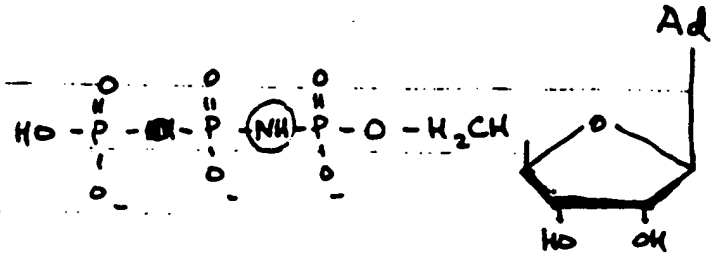
This system would tend to localize the oligonucleotide in liver; since this is the major clearing site for asialoglycoproteins.

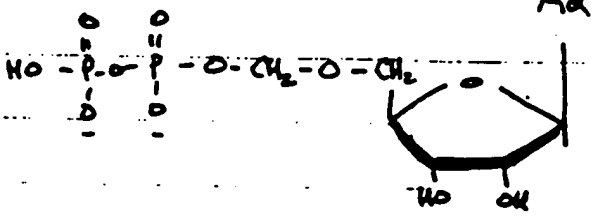
I have been trying to think of an acronym for Specific Translation Inhibiting Oligonucleotide Phosphotriester. The only one which seems reasonable is "STOP" molecule. I think I'll try using that abbreviation form – tell me what you think?

The use of a 2'–5' linkage in place of 3'–5' phosphodiester link seems very interesting. This is the type of linkage produced in cells exposed to interferon. They make oligomers like HO – A 2'– p – 5' A 2'– p – 5' A ppp (an oligoribonucleotide)

Some new technology would be required to chemically synthesize 2′→5′ oligoribonucleotides. I suspect that would take 3–5 years to really work out the details. You might be able to get the basic process in a year or two. If an enzyme exists which does this the process could be much faster.

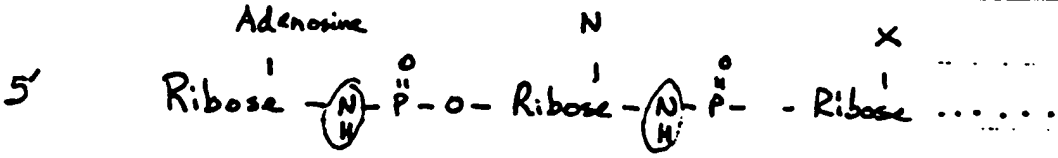
I just had another thought regarding stable internucleotide linkages. Perhaps one could substitute another linking atom for phosphorous or oxygen analogous to the use of amides in ATP to prevent breakdown (also methylene ether linkages)





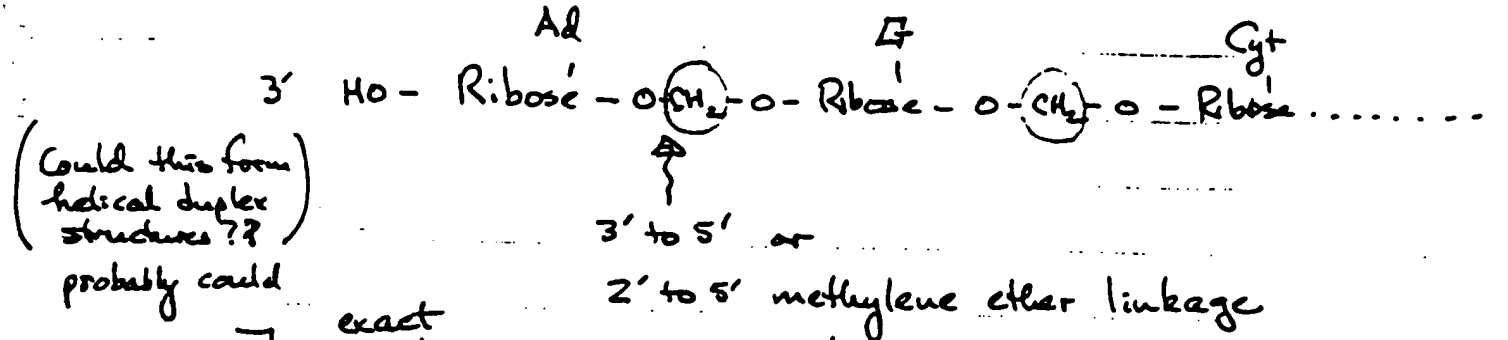
"Adenosine triphosphoramidate" "α methylene ADP"

The analogous structures would be an oligomer



"Oligonucleotide phosphosamidate"

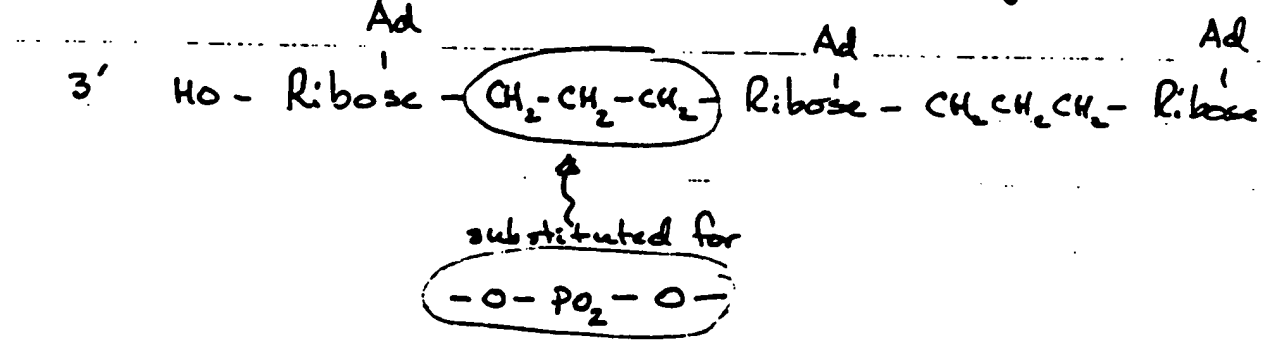
or



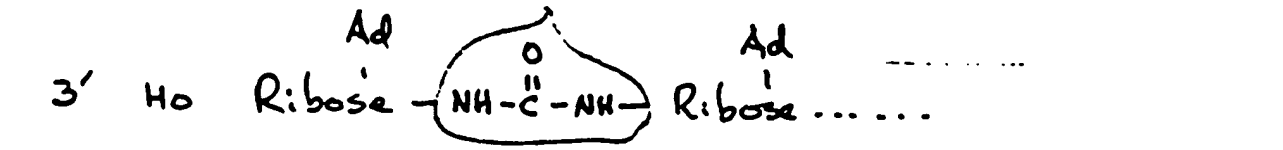
(Could this form helical duplex structures?? probably could

This exact structure is not known to occur in nature as far as I am aware. I'm not sure how chemically stable it would be but it looks OK to me. Clearly it is uncharged and therefore less soluble than the phosphoramide or the 2′→5′ phosphodiester.

You might also be able to make a methylene bridged structure of this type where CH_2 is substituted for oxygen and phosphorous in normal linkage



The last one for today is an amide bridge substitute for the phosphodieste



this would be more polar and thus more soluble than the methylene or methyl diether form

Since polypeptides can form α helical structures it is likely that this guy would also form duplex structures. It might be more labile in a cell than some of the other forms since the amide bond could possibly be attacked by nonspecific proteases.

Aside from the type of different internucleotide linkages which are possible in the "STOP molecule", one question which has arisen is the antigenicity of polynucleotides. By themselves oligonucleotides are very poorly antigenic. The only known autoimmune disease associated with anti-DNA antibodies is systemic lupus erythematosis. However it is possible to raise antibodies to mononucleotides coupled to serum albumin. Thus this problem is possible but unlikely to present a serious objection to the use of STOP.

One question we raised that needs to be answered quantitatively is what is the rate of reaction of STOP with an RNA (ie how fast could it begin to block specific mRNA translation) A related question is how much material would have to be injected to inactivate say 1 Kg of virus mRNA in 24 hours?

In order to calculate a rate of reaction of an oligonucleotide of say 20 NT length with an mRNA one can use the formula

$$K_{STOP} = \frac{2 \times K_{coli} \times \text{Genome Complexity (coli)}}{\text{STOP complexity}} \left[\frac{450}{20}\right]^{-\frac{1}{2}}$$

(450: length of coli DNA standard; 20: length of STOP)

$K_{coli} = 0.25\ M^{-1} sec^{-1}$ (second order rate constant)
Length coli = 450 NT for this rate in 0.12 M salt
$G_{coli} = 4.2 \times 10^6$ NT (NT = base pairs of NT's in coli genome)
K_{STOP} = reassoc. rate constant expected
STOP = 20 NT complexity = 20 NT length

$$K_{STOP} = \frac{2 \times 0.25 \times 4.2 \times 10^6}{20} \left[\frac{20}{450}\right]^{\frac{1}{2}} = 2.21 \times 10^4\ M^{-1} sec^{-1}$$

In an RNA or ss DNA driven reaction K is pseudofirst order and has the same value as above Under these circumstances $K = \frac{\ln 2}{C_o t_{\frac{1}{2}}}$

Where C_o = initial & final concentration of STOP and $t_{\frac{1}{2}}$ is the reaction half time. Thus $C_o t_{\frac{1}{2}} = 3.13 \times 10^{-5}$ (M sec). for STOP reacting with an mRNA. If we were to achieve a reasonable intracellular concentration of STOP of say 5 μg/ml of cell volume how fast would this reaction take place?

Or put another way how long would it be before 75% of the mRNA target was complexed with STOP as a hybrid.
(75% rxn is 2 half lives or $2 \times C_o t_{1/2}$)

ave NT molecular weight = 315

5 μg/ml = 15.9 μmoles/l = 16 μM in NT
(by convention you calculate the concentration of an oligomer or DNA in moles NT/liter

C_o = 16 μM

$C_o t_{1/2} = 3.13 \times 10^{-5}$ M sec

∴ $t_{1/2}$ = 1.96 seconds or 4 seconds to 75% reaction

This is clearly fast enough to do the job.

The related question of how much viral mRNA can be inactivated by that amount of STOP molecule. To get 5 μg/ml in all cells (assuming uniform distribution) you would need to inject about 500 mg/100 kg body weight (ie ~ ½ gm ST

500 mg STOP = 79 μmoles STOP (20 NT long)

Theoretically 1 molecule of STOP can inactivate 1 molecule of mRNA. Most mRNA's are around 1000 NT long. Thus 500 mg STOP-20 could inactivate 25 gms of viral mRNA.

Since in general there is about 10 x more viral mRNA than DNA, we would expect 1/10th that amount of viral DNA to be blocked. However a viral genome is about 3×10^5 NT long or about 300 times the size of the viral mRNA. Therefore 500 mg STOP could block the replication of 750 gms of viral DNA

$$25 \text{ gm} \times \left(\frac{1}{10}^{th}\right) \times 300 = 750 \text{ gms virus}$$

This is far in excess of the amount of viral DNA one might expect to find in an infected human.

I would estimate from this that the minimum effective dose would be much less - at a guess about 1 gm of virus. At this level the required dose of "STOP-20NT" would be 0.7 mg. For a ten fold excess lets say 10 mg. The 75% inactivation time would then be 3.3 minutes (theoretically).

How much could this cost? If we use current retail cost figures as of last August 2 mg of 15 NT oligomer cost ₱8500 while 1 mg cost retail ₱7500. I am sure this is exorbitant but from the difference between 1 and 2 mg of ₱100.00 I would guess that the actual cost in materials is close to ₱50 per mg. Since this technique is now automated

that is probably in excess of the real cost. However, using that figure a dose of 10mg would cost $500.00 Bulk production could probably cut that to $50 which would be reasonable.

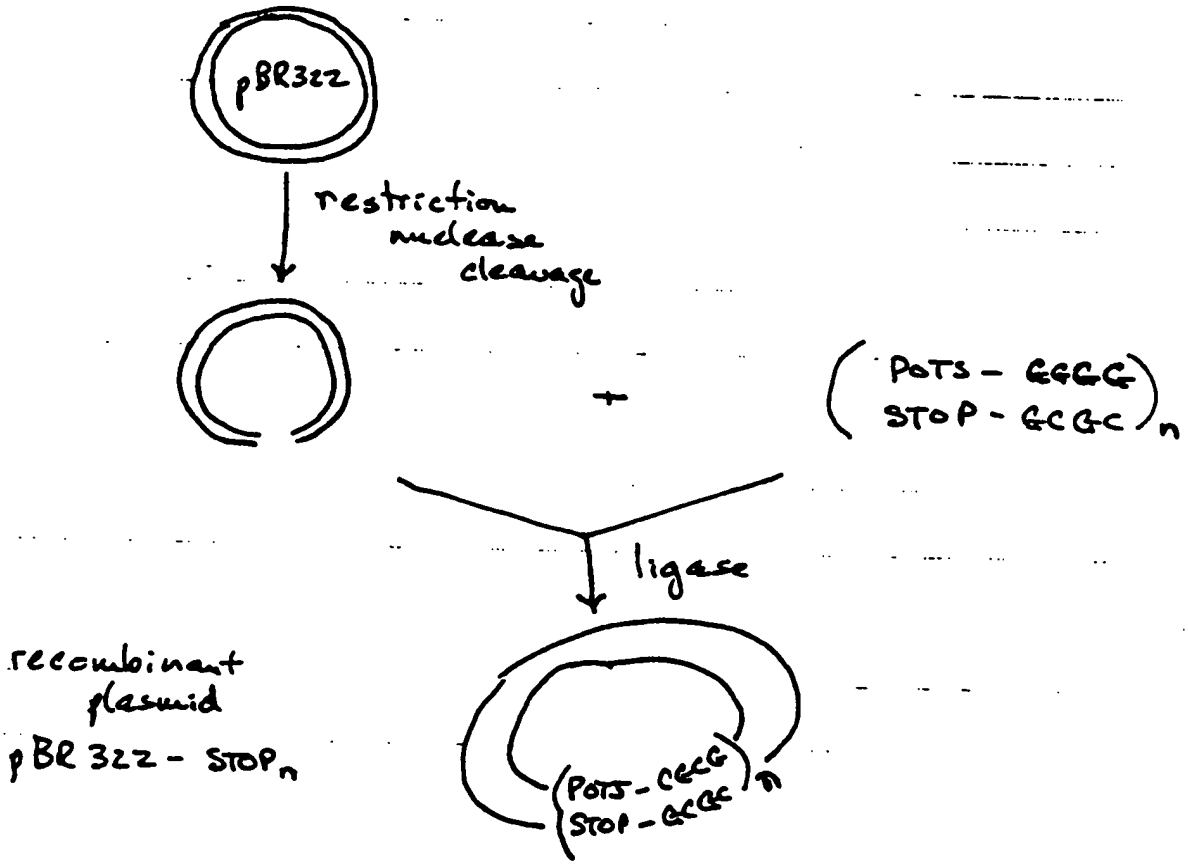
I have thought about ways to produce this material using recombinant technology. The method would be to synthesize a STOP polymer by blunt end ligation using polynucleotide ligase. The trick would be to leave a restriction nuclease cleavage site at the end of each STOP molecule (eg GCGC) [GCGC is the Cleavage sequence for nuclease Hha I (and Hae II)]

STOP-GCGC $\xrightarrow{\text{ligase}}$ STOP-GCGC-STOP-GCGC-ST
(STOP-GCGC)$_n$

This molecule in its double stranded form would be

(POST-CGCG-POTS-GCGC.....
STOP-GCGC-STOP-GCGC.....)

This can be condensed with a high producing plasmid such as pBR 322 to yield a recombinant shown on next page

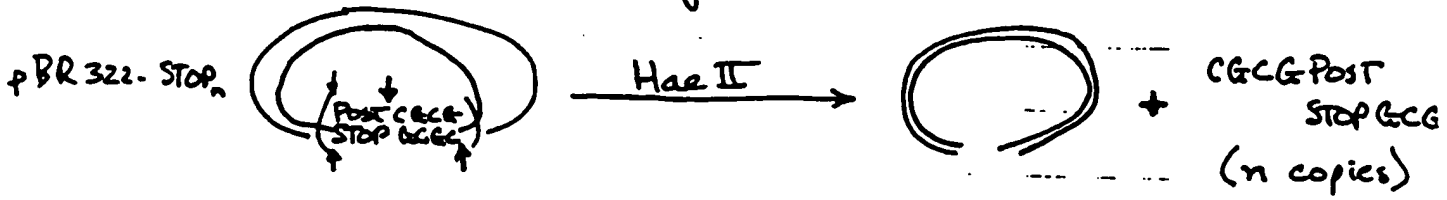


The plasmid pBR322 - STOP$_n$ could then be cloned as usual and large amounts grown in E. coli. To isolate STOP the procedure would be as follows:

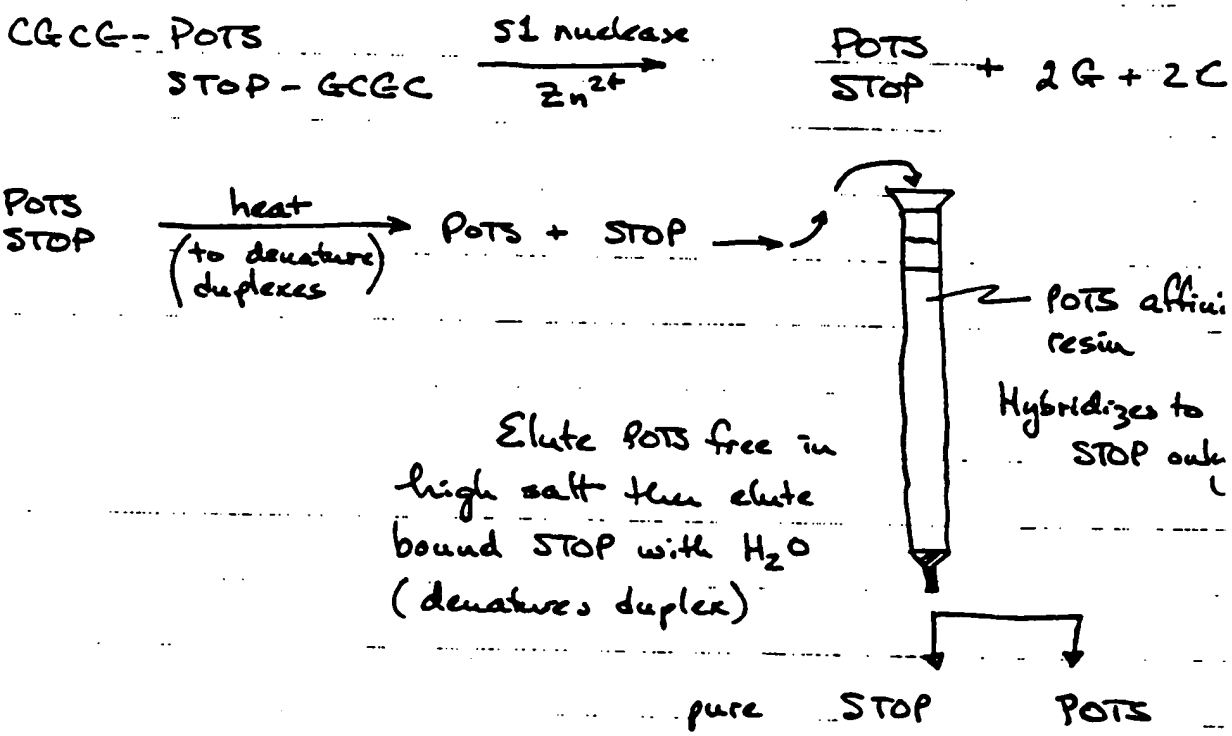
Std. technique

① Isolate and purify pBR322 - STOP$_n$

② Cleave with restriction nuclease Hae II or Hha I. The best choice will usually be Hae II which will do this:



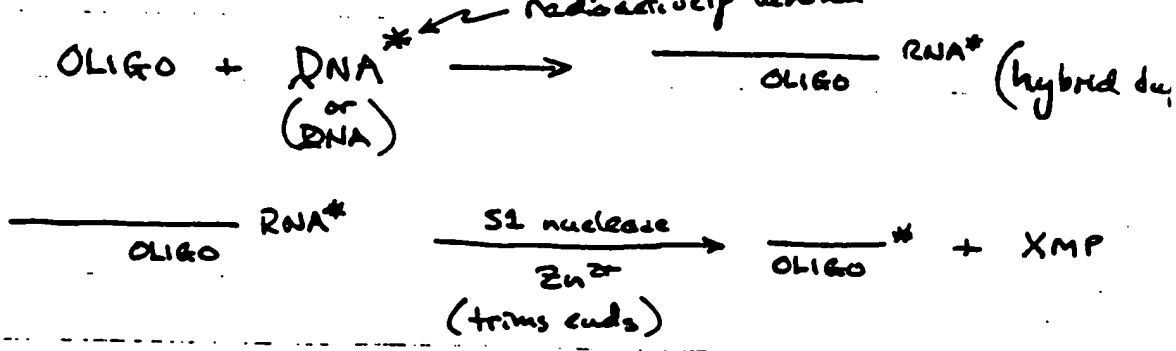
③ Isolate STOP— on a POTS - Sepharose affinity coloma after removal of GCGC tail by S1 nuclease treatment (specific for single stranded DNA.



The cost of STOP produced essentially in this manner (Note: there must be a triester formation step just before denaturing $\frac{POTS}{STOP}$ above) would be analogous to the cost of producing insulin via recombinant bugs. That cost is about 1¢/mg. Since in a cell there is generally speaking 10-100x more protein than DNA a rough estimate of the cost would be at most about 10^{00}/mg STOP. Thus a 10mg treatment dose would cost ≤ 10^{00}/10mgs STOP.

(Not bad for off the top so far, eh?)

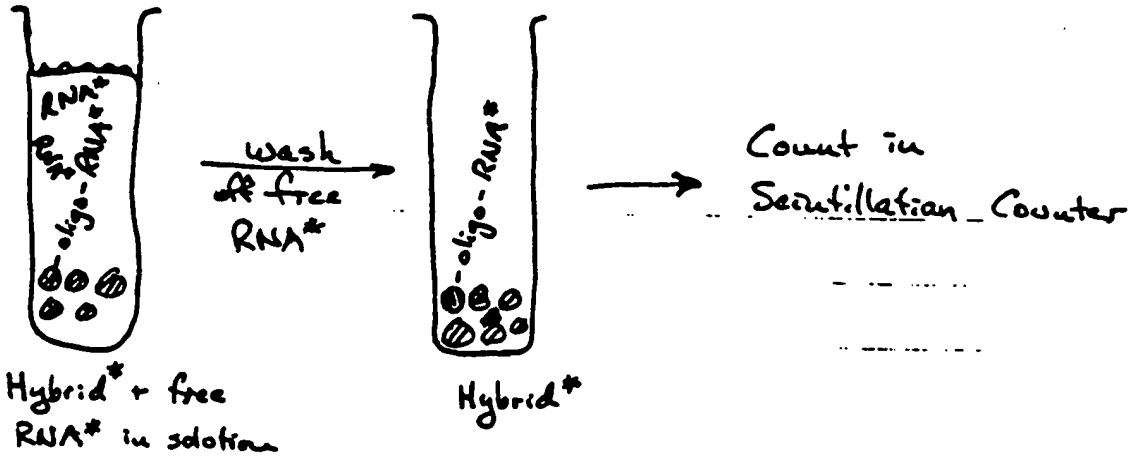
The idea I had for using oligonucleotides to detect specific microorganisms (eg Gonococcus) is analogous to radioimmunoassay. The basic principle is that a hybrid can be specifically formed between an oligonucleotide and a particular DNA or RNA species, which is absolutely specific to that particular sequence. This can be competed out by unlabeled DNA or RNA of the same sequence only.



The hybrid can be measured by binding to DEAE paper or hydroxylapatite chromatography.

In the presence of cold, unlabeled specific RNA (or DNA) the oligonucleotide would be competed for in forming hybrids. Thus less radioactive hybrid would be formed. The degree of reduction would be a measure of the amount of competing RNA (or DNA) present, thus allowing estimation of the amount of organism present in say a vaginal smear or sputum sample.

The calculations for the rate of reaction, sequence excess required, sensitivity, cost and complication are rather involved so I would like to put that off to a future letter. However, one last note on this and then I'll quit. The reaction could be run using oligomer bound to a solid support (eg Glass bead ↔ oligomer) in a rapid reaction system known as PERT (phenol emulsion reassociation technique) which is 10,000 times faster than normal solution hybridizations and does not require purification of DNA prior to use. The idea would then be to put the sample directly into the phenol emulsion in a small tube containing glass bead → oligo + RNA* and shake for an hour or so. The beads could then be rinsed several times with saline and counted directly in a scintillation counter



I know thats a lot of stuff, but you did say to put down my thoughts so there they are. I'll provide more detail later. I hope this will do for a start. I am also including a bibliographic search done on "synthetic oligonucleotides" in Bio Abstracts and copies of some articles for a recent Science which may interest you regarding oligonucleotides.

If you plan a coming to San Diego soon let me know so we can get together and talk. Otherwise I'll be in touch when I can.

Best regards

Rich

EXHIBIT III

INFORMATION SHEET

Date: _____ Docket No. 20886

Attorn y GGK/ WMS Ref. by Drew Senyei

CLIENT: TULLIS, RICHARD H. Phone (714) 454-8571

741 Arenas Street

La Jolla, California 92037 Phone _____

Phone _____

INVENTORS: _____ Citizen _____

Phone _____

Citizen _____

Phone _____

SUBJECT MATTER: SYNTHETIC OLIGONUCLEOTIDES/ ANTI VIRAL AGENTS

WORK ORDERED: REVIEW AND ADVISE RE MATERIALS RECEIVED

DATA RECEIVED: letter with articles

NOVELTY DATA: First Public Disclosure: -

First Placed On Sale: -

First Sold: - First Used: -

Explanation: _____

PRIORITY DATA: Conception: - Reduction to Practice: -

Explanation: _____

COST: Estimate: _____ Advance: _____

ASSIGNMENT: _____

REMARKS: _____

Xer x/~~copy~~ me

CARDS - for GGK & WMS FULWIDER, PATTON, RIEBER, LEE & UTECHT

je + me

J

EXHIBIT IV

DEPARTMENT OF COMMUNITY MEDICINE
SCHOOL OF MEDICINE

LA JOLLA, CALIFORNIA 92093

11/6/80

Dear Bill

Here are draft copies of our disclosures for both and the STOP antiviral oligonucleotide.

Fire away.

Hope to hear from you soon.

Phil

P.S. I'm checking with Kentron today re: contract work on the Rigoher clinical test system.

EXHIBIT V

California Biomedical Research Foundation
DEPARTMENT OF BIOLOGICAL CHEMISTRY
741 Arenas Street
La Jolla, California 92037
Telephone (714) 454-8571

4 Feb 81

Dear Bill,

Here is my first draft of the STOP patent. I think at this point that it reads well enough to be read through, although much remains to be done. I have still not described in detail the other types of STOP polymers and their potential production. Other things like that remain. I hope this has enough of the meat to proceed.

Otherwise will expect to talk to you in a few days

Thanks,

Rick

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SANTA BARBARA • SANTA CRUZ

DEPARTMENT OF COMMUNITY MEDICINE
SCHOOL OF MEDICINE

LA JOLLA, CALIFORNIA 92093

EXHIBIT VI

23 Feb 1981

Dear Bill,

Here are some more of the relevant papers on the STOP polymers.

Hope to hear from you soon

Rick